INFECTION AND IMMUNITY, Oct. 1996, p. 4401–4405 Vol. 64, No. 10
0019-9567/96/$04.00+0


# Attenuated Virulence of Uridine-Uracil Auxotrophs of *Aspergillus fumigatus*

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Received 13 May 1996/Returned for modification 2 July 1996/Accepted 30 July 1996

***Aspergillus fumigatus*** **mutants that are deficient in the de novo UMP biosynthesis pathway because of a mutation in the *pyrG* gene encoding orotidine-5′-phosphate decarboxylase (and therefore auxotrophic for uridine or uracil) were evaluated in a murine model of invasive aspergillosis. These mutants were entirely nonpathogenic, and mutant conidia remained ungerminated in alveolar macrophages. Both the germination and virulence defects could be restored by supplementing the drinking water of the animals with uridine. DNA-mediated transformation of one of the *pyrG* mutants with the *Aspergillus niger pyrG* gene also restored virulence. These results suggest that uridine and uracil are limiting in the lung environment, thus preventing conidium germination and hence virulence of the *pyrG* mutants.**

To date, no specific factor has been conclusively shown to be involved in the pathogenicity of the opportunistic fungal pathogen *Aspergillus fumigatus*, which often causes fatal pulmonary invasive aspergillosis in patients with acute leukemia or other hematological malignancies and in organ transplant recipients (1). In particular, the analysis of genetically engineered mutants that are unable to produce secreted proteases shows that they are not essential for the establishment of invasive aspergillosis in immunocompromised mice (10, 16, 25, 26). However, the analysis of mutants of both *A. fumigatus* and *Aspergillus nidulans* suggested that some specific biosynthetic pathways are essential for the development of the fungus in the host tissue. For example, *p*-aminobenzoic acid-requiring mutants of these two species are nonpathogenic in murine models of invasive aspergillosis. This defect can be corrected by supplementing the drinking water of the animals with *p*-aminobenzoic acid (19, 27), suggesting that the concentration of *p*-aminobenzoic acid is too low to allow the growth of these *Aspergillus* species in vivo.

In the course of developing a new DNA-mediated transformation system for *A. fumigatus*, we have isolated *pyrG* mutants of *A. fumigatus* (4). These mutants are auxotrophic for uridine and uracil and carry a mutation in the *pyrG* gene encoding orotidine-5′-phosphate decarboxylase that catalyzes the last step of de novo UMP biosynthesis. Although the pyrimidine biosynthesis pathway has been implicated in the in vivo proliferation of several bacterial and fungal pathogens (6, 11, 15, 17), it has never been shown to participate in *A. fumigatus* pathogenicity. Results presented in this paper show that *pyrG* mutants of *A. fumigatus* have an attenuated virulence in a murine model of pulmonary invasive aspergillosis. Histopathological analysis at the onset of infection suggests that this defect is related to a reduced level of conidial germination within the alveolar macrophage that is likely to be due to limiting levels of uridine and uracil.

The following murine model of invasive aspergillosis was used to study the virulence of *A. fumigatus* strains. White female mice (SPF OF1; weighing 16 to 18 g; IFFA CREDO, L'Arbresle, France) were immunosuppressed by intraperitoneal injection of cortisone acetate (250 mg · kg of body weight$^{-1}$) 3 days prior to infection and on day 0. Conidium inocula were prepared by growing *A. fumigatus* strains on complete medium (3) supplemented with 5 mM uridine and uracil for a week at 37°C and harvesting the conidia in phosphate-buffered saline (PBS)–Tween 20 (0.1%). On day 0, mice were anesthetized by the inhalation of diethyl ether and inoculated by inhalation of a droplet of 50 μl of PBS-Tween 20 (0.1%) containing $10^6$ conidia. Immunosuppression was prolonged by cortisone acetate injections (250 mg · kg$^{-1}$) on days 2 and 4 only since a longer immunosuppression regimen resulted in the occurrence, after 10 days of infection, of an elevated mortality in mice that had received a control inoculum (50 μl of PBS-Tween 20 [0.1%]). *A. fumigatus* CBS144-89, CEA17, CEA22, and CEA17-14/3 (see below for description) were tested four times, three times, twice, and once, respectively, with cohorts of 10 animals. The pathogenicity of *A. fumigatus* CEA17 in mice receiving uridine (100 mM) in their drinking water was tested three times with cohorts of 10 animals. The number of dead mice was counted daily. Lungs were removed from dead animals or from animals that were sacrificed 8, 24, or 48 h postinoculation and either homogenized in saline or fixed in 10% Formol-saline or Kryofix (Merck, Nogent sur Marne, France) for histological examination. Lung homogenates were plated on complete medium (3) containing 5 mM uridine and 5 mM uracil when required, and the recovered fungal clones were purified to single colonies that were tested for uridine or uracil auxotrophy. Fixed lungs were embedded in paraffin, and serial tissue sections (6 to 7 μm) were stained with Grocott silver stain by standard techniques (9).

To monitor the germination of the prototrophic and auxotrophic strains at the onset of infection, 28- to 30-g white female mice (SPF OF1; IFFA CREDO) were subjected to the immunosuppressive regimen described above and inoculated with $10^8$ conidia. After 0, 8, 18, 24, and 42 h, two to three animals per cohort were anesthetized by subcutaneous injection of 5 ml of a 1:1 mixture of midazolam kg$^{-1}$ (Dormicum; Roche Inc., Basel, Switzerland) and fentanyl-fluanisone (Hypnorm; Janssen Pharmaceutica, Beerse, Belgium) (7) and subjected to a bronchoalveolar lavage by intratracheal instillation of 1 ml of sterile saline. Cells in the bronchoalveolar lavage were pelleted by centrifugation and subjected to osmotic lysis

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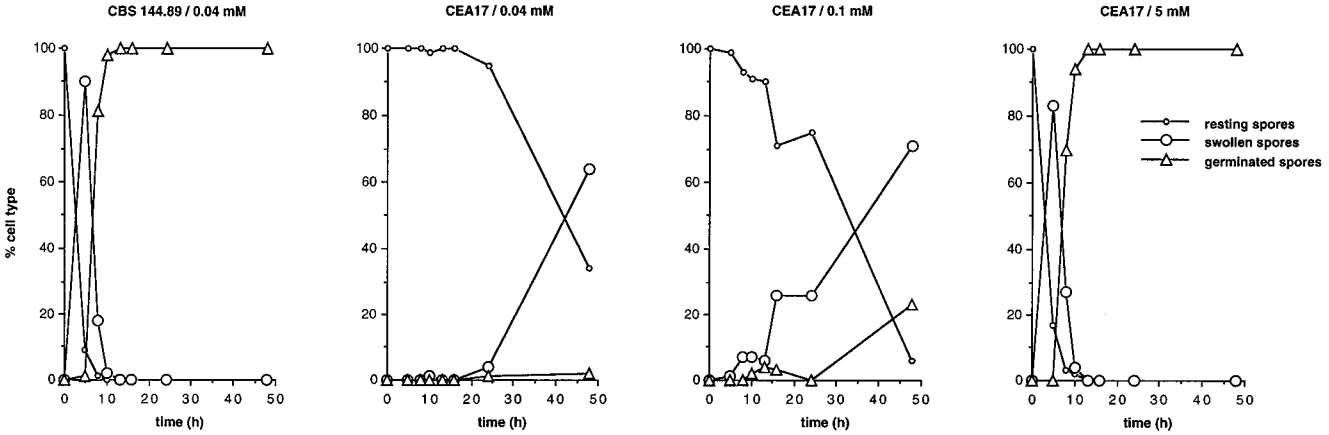


FIG. 1. Kinetics of conidium germination of the *A. fumigatus* wild-type (CBS144-89) and *pyrG* (CEA17) strains in complete medium supplemented with varying concentrations of both uridine and uracil. Slides coated with complete medium were spot inoculated with $5 \times 10^3$ conidia of the wild-type or mutant strain and incubated at 37°C. At the indicated times, slides were examined microscopically and the percentages of resting, swollen, and germinated conidia were estimated.

in order to remove lung cells. The percentage of germinated conidia among the recovered fungal cells was then estimated by microscopic examination of the sample.

**Ex vivo germination of *A. fumigatus pyrG* strains.** *A. fumigatus* CEA17 and CEA22 are uridine-uracil-auxotrophic derivatives of the wild-type pathogenic isolate CBS144-89. They were isolated by virtue of their resistance to 5-fluoro-orotic-acid (10 mg/ml). Whereas CEA17 was obtained following low-dose chemical mutagenesis (4), CEA22 is a spontaneous uridine-uracil auxotroph. Both strains were shown to carry a mutation in the *pyrG* gene by the fact that uracil-uridine prototrophs could be recovered after transformation with a plasmid carrying the *Aspergillus niger pyrG* gene (reference 4 and data not shown). Analysis of the radial growth of strains CEA17 and CEA22 after 48 h at 37°C in complete medium (3) containing various amounts of uridine and/or uracil revealed that both mutants require high levels of both uridine (>3.0 mM) and uracil (>3.0 mM) to sustain a growth that is about 85% of that of the wild-type strain (data not shown). Conidium germination was monitored by microscopic examination of slides coated with complete medium (3) supplemented with varying concentrations of uridine and uracil and spot inoculated with ca. $5 \times 10^3$ freshly harvested conidiospores. As shown in Fig. 1, uridine-uracil deprivation resulted in a low rate of conidium swelling and in the inability of the mutant conidia to produce germ tubes. This, together with the requirement for high levels of uridine and uracil to reach wild-type growth (which contrasts with the lower uridine-uracil requirements of *Saccharomyces cerevisiae* or *Candida albicans* orotidine-5′-phosphate decarboxylase mutants [0.1 mM] [2, 24]), suggests that very low affinity uridine and uracil carriers are present in *A. fumigatus*, as previously proposed for *A. nidulans* (18, 20).

**Avirulence of *A. fumigatus pyrG* strains.** The virulence of *pyrG* strains CEA17 and CEA22 was compared with that of wild-type strain CBS144-89 in our murine model of invasive aspergillosis. While infection with the wild-type strain resulted in an average mortality of 84% after 10 days, mortality in mice inoculated with either uridine or uracil auxotrophs averaged 8%, similar to what was observed when mice were inoculated with sterile PBS-Tween only (Fig. 2 has an example) and likely to be due to opportunistic bacterial infections.

The analysis of bronchoalveolar lavages obtained at different times during the course of the infection showed that while a significant rate of germination of wild-type conidiospores was

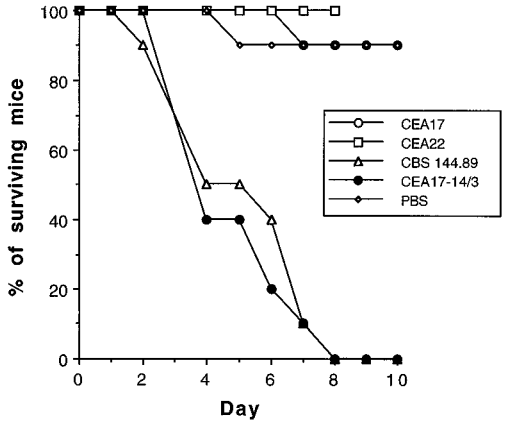


FIG. 2. Survival of mice inoculated intranasally with $10^6$ conidia of *A. fumigatus* CBS144-89 (PyrG$^+$), CEA17 (PyrG$^-$), CEA22 (PyrG$^-$), and CEA17-14/3 (PyrG$^+$) or with 50 μl of PBS-Tween 20 (0.1%) (PBS). Each group consisted of 10 animals that had been immunosuppressed with cortisone. Data presented are representative of several replicate experiments except for strain CEA17-14/3, which was tested only once.

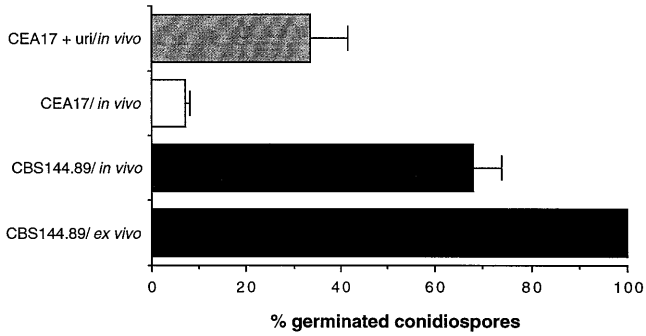


FIG. 3. Percentages of germinated conidia found after 24 h of infection in bronchoalveolar lavages of immunosuppressed mice inoculated intranasally with $10^8$ conidia (in vivo) of *A. fumigatus* CBS144-89 (PyrG$^+$) or CEA17 (PyrG$^-$) or after 24 h of incubation at 37°C on slides coated with complete medium (ex vivo). Uridine (100 mM) was added to the drinking water of animals where indicated (+ uri). Values are the means of the results obtained with two or three mice, and standard deviations are indicated.

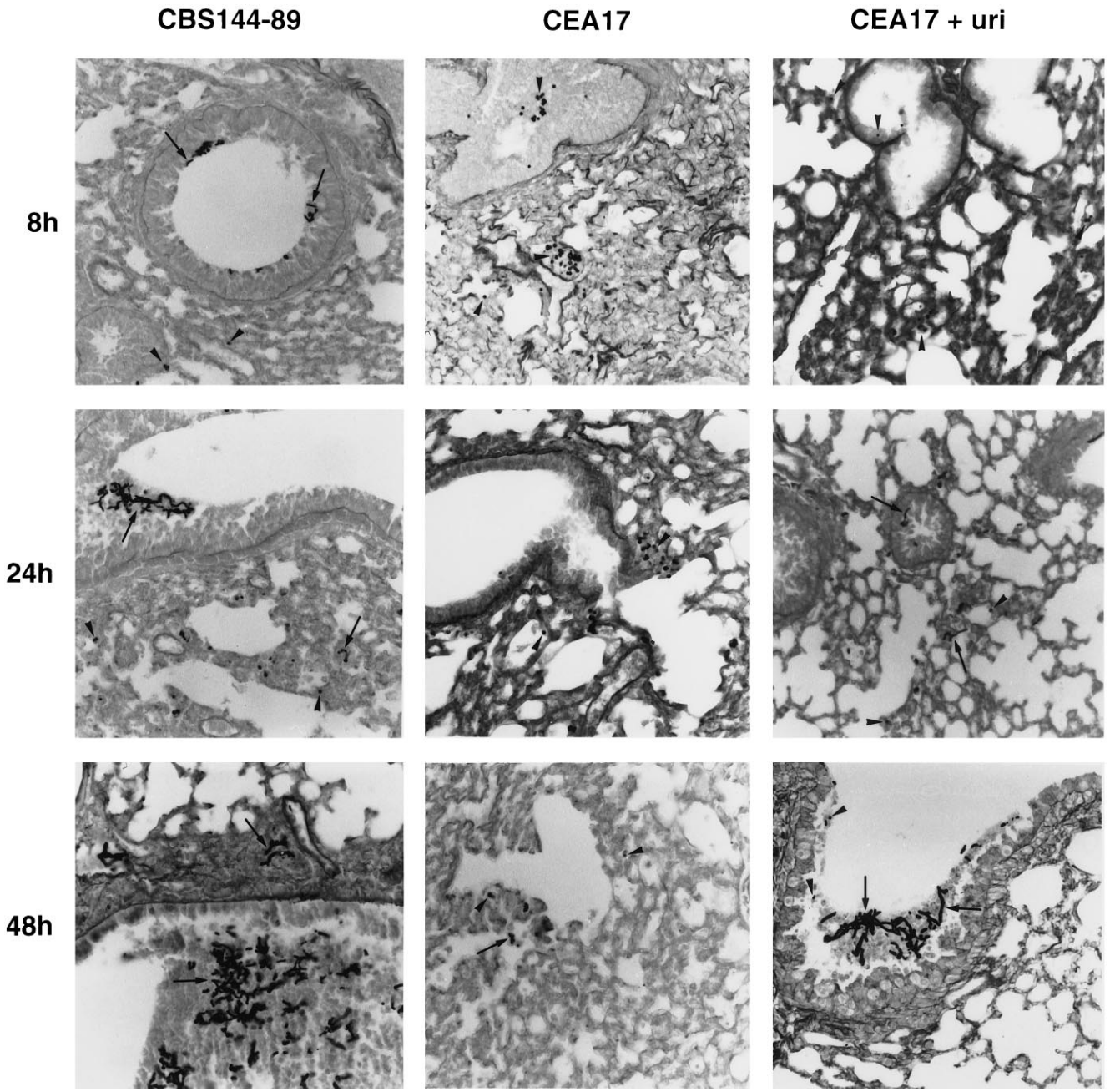


FIG. 4. Paraffin sections from the lungs of immunosuppressed mice inoculated with *A. fumigatus* CBS144-89 ($PyrG^+$) or CEA17 ($PyrG^-$) and sacrificed after 8, 24, or 48 h of infection. Sections were stained with Grocott's silver stain. Fungal elements stain black, and examples of conidia and hyphal elements are indicated by arrowheads and arrows, respectively. Uridine (100 mM) was added to the drinking water of animals where indicated (+ uri). Each picture is representative of the results obtained with six mice. Magnification, ca. ×50.

achieved within the first 24 h following challenge, mutant conidia remained mostly ungerminated after 24 h (Fig. 3) or 42 h (data not shown) of infection. Furthermore, we noticed that conidia could be easily recovered at all times from the lungs of animals infected with the auxotrophic strain whereas fungal elements were hardly recovered after 24 h of infection from mice inoculated with the wild-type strain, suggesting that in the latter case germlings were in close association with lung tissues.

These results were confirmed by histological analysis of lungs obtained from animals infected with the wild-type or mutant strains and sacrificed early during the course of the infection. As shown in Fig. 4, wild-type conidia were found in the bronchial tubes and alveolae and had reached a substantial level of germination after 8 h of infection. At later time points, mycelial invasion of the bronchial epithelium and alveolae could be easily observed. In contrast, conidia of the uridine-uracil auxotroph were mostly ungerminated after 48 h of infection (Fig. 4) and remained frequently associated with alveolar macrophages.

**De novo UMP biosynthesis and in vivo conidium germination.** In order to rule out the occurrence of a mutation, other

than the *pyrG* mutation, that could account for the avirulence phenotype of strain CEA17, we tested the pathogenicity of strain CEA17-14/3, a derivative of CEA17 that carries a random genomic insertion of the cloned *A. niger pyrG* gene and is therefore prototrophic for uridine and uracil (4). As shown in Fig. 2, CEA17-14/3 was as virulent as the wild-type strain. *A. fumigatus* strains recovered from the lungs of infected animals showed hybridization patterns similar to that obtained with CEA17-14/3 when the *A. niger pyrG* gene was used as a probe (data not shown), suggesting that they are identical to CEA17-14/3 and that they had not undergone any genomic reorganization that could account for their virulence. Therefore, the *pyrG* mutation alone is responsible for the avirulence of strain CEA17, demonstrating that de novo UMP biosynthesis is required for germination of *A. fumigatus* conidia in the lung.

This result was confirmed by data obtained when mice infected with strain CEA17 were given uridine (100 mM) in their drinking water 3 days prior to inoculation and during the course of the infection. Mortality was then much higher (37% after 10 days of infection) than that obtained when strain CEA17 was inoculated into mice that did not receive uridine in their drinking water (8%, see above). Fungi recovered from the lungs of infected animals were all dependent on uridine or uracil for growth. In addition, a significant increase in the rate of conidium germination could be observed either by analysis of bronchoalveolar lavage (Fig. 3) or by histological analysis of lungs recovered from animals that received uridine in their drinking water and that had been inoculated with strain CEA17. As shown in Fig. 4, most conidia remained ungerminated after 8 h of infection, but numerous germlings and filaments could be observed after 24 and 48 h of infection. Taken together, these data suggest that uridine and uracil are limiting in the lung environment, thus preventing *pyrG* strains from germinating and establishing invasive aspergillosis. Nevertheless, as shown ex vivo (see above), in vivo uridine supplementation is not sufficient to ensure a wild-type growth rate and therefore wild-type virulence.

Our data, together with those of others (5, 8, 14, 22, 23) suggest that, during invasive aspergillosis, wild-type conidia are predominantly phagocytized by alveolar macrophages and that, because of the reduced functionality of the phagocyte resulting from the immunosuppressive treatment (21, 22), they are able to escape killing, germinate, and reach the external milieu. The observation that after 48 h of infection most of the *pyrG* mutant conidia remain ungerminated within alveolar macrophages is in close agreement with this hypothesis. The *pyrG* mutants show a germination defect both ex vivo and in vivo, and the intraphagosomal concentrations of uridine and uracil do not appear to be sufficient to sustain growth of the mutants. Limiting levels of uridine and uracil in tissues, especially in the phagolysosomal compartment and in the external milieu, have already been proposed to explain data obtained with other pathogens which, when defective in the de novo UMP biosynthesis pathway, show reduced virulence. For example, *Salmonella typhimurium carAB* mutants deficient in the first step of de novo UMP biosynthesis because of the lack of carbamoyl phosphate synthase activity are avirulent (12) and *C. albicans ura3* mutants that lack orotidine-5′-phosphate decarboxylase show reduced virulence in topical and systemic models of topical and systemic candidiasis (2, 11).

The conditional virulence of the *A. fumigatus pyrG* mutants that can be modulated by varying the levels of available uridine will represent a powerful tool to understand the interactions of the conidia with the host cells either in vivo or in cellular assays that mimic some steps of invasive aspergillosis. It may also be useful to understand the persistence of *A. fumigatus* within the lung. In addition, the efficient virulence restoration in the *pyrG* strain by DNA-mediated transformation with the *A. niger pyrG* gene may lead to the development of an in vivo expression technology system similar to that developed for *S. typhimurium* (12). In vivo expression technology relies on the complementation of the virulence defect of a *S. typhimurium* strain that has a mutation in a biosynthetic gene by operon fusions to this gene. It has been used successfully to identify genes that are specifically expressed during infection and which participate in the infection process (12, 13). Similarly, when gene fusions to the *A. niger pyrG* gene in a *pyrG* strain of *A. fumigatus* are used, in vivo expression will confer virulence whereas ex vivo expression will be easily monitored since it confers uridine or uracil prototrophy or 5-fluoro-orotic acid sensitivity. Construction of a shuttle vector carrying a transformation marker for *A. fumigatus* (e.g., hygromycin resistance [16]) and a promotorless *A. niger pyrG* gene should therefore facilitate the identification of *A. fumigatus* sequences that drive *pyrG* expression during infection only. The analysis of such sequences will undoubtedly help our understanding of *A. fumigatus* virulence.

Thanks are due to S. Paris, A. Pugsley, and P. Sansonetti for critical reading of the manuscript and to M. Cormier for her expertise in typing the paper.

## REFERENCES


1. **Cohen, J.** 1991. Clinical manifestations and management of aspergillosis in the compromised patients, p. 117–152. *In* D. W. Warnock and M. D. Richardson (ed.), Fungal infection in the compromised patient, 2nd ed. John Wiley & Sons, New York.
2. **Cole, M. F., W. H. Bowen, X.-J. Zhao, and R. L. Cihlar.** 1995. Avirulence of *Candida albicans* auxotrophic mutants in a rat model of oropharyngeal candidiasis. FEMS Microbiol. Lett. **126:**177–180.
3. **Cove, D. J.** 1966. The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. Biochim. Biophys. Acta **113:**51–56.
4. **d'Enfert, C.** 1996. Selection of multiple disruption events in *Aspergillus fumigatus* using the orotidine-5′-decarboxylase gene, *pyrG* as a unique transformation marker. Curr. Genet. **30:**76–82.
5. **Epstein, S. M., E. Verney, T. D. Miale, and H. Sidransky.** 1967. Studies on the pathogenesis of experimental pulmonary aspergillosis. Am. J. Pathol. **51:**769–788.
6. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83:**5189–5193.
7. **Flecknell, P. A., and M. Mitchell.** 1984. Midazolam and fentanyl-fluanisone: assessment of anaesthetic effects in laboratory rodents and rabbits. Lab. Anim. **18:**143–146.
8. **Gernez Rieux, C., C. Voisin, C. Aerts, F. Wattel, and B. Gosselin.** 1967. Experimental aspergillosis in the guinea pig. Dynamic study of the role of alveolar macrophages in the defense of the respiratory tract, after massive inhalation of *Aspergillus fumigatus* spores. Rev. Tuberc. Pneumol. **31:**705–725.
9. **Grocott, R. G.** 1955. A stain for fungi in tissue sections and smears using Gomori methenamine silver nitrate technique. Am. J. Clin. Pathol. **25:**975–979.
10. **Jaton-Ogay, K., S. Paris, M. Huerre, M. Quadroni, R. Falchetto, G. Togni, J. P. Latgé, and M. Monod.** 1994. Cloning and disruption of the gene encoding an extracellular metalloprotease of *Aspergillus fumigatus*. Mol. Microbiol. **14:**917–928.
11. **Kirsch, D. R., and R. R. Whitney.** 1991. Pathogenicity of *Candida albicans* auxotrophic mutants in experimental infections. Infect. Immun. **59:**3297–3300.
12. **Mahan, M. J., J. M. Slauch, and J. J. Mekalanos.** 1993. Selection of bacterial virulence genes that are specifically induced in host tissues. Science **259:**686–688.
13. **Mahan, M. J., J. W. Tobias, J. M. Slauch, P. C. Hanna, R. J. Collier, and J. J. Mekalanos.** 1995. Antibiotic-based selection for bacterial genes that are specifically induced during infection of a host. Proc. Natl. Acad. Sci. USA **92:**669–673.
14. **Merkow, L. P., S. M. Epstein, H. Sidransky, E. Verney, and M. Pardo.** 1971. The pathogenesis of experimental pulmonary aspergillosis. An ultrastructural study of alveolar macrophages after phagocytosis of *A. flavus* spores in vivo. Am. J. Pathol. **62:**57–74.
15. **Mintz, C. S., J. Chen, and H. A. Shuman.** 1988. Isolation and characterization of auxotrophic mutants of *Legionella pneumophila* that fail to multiply in human monocytes. Infect. Immun. **56:**1449–1455.

16. **Monod, M., S. Paris, J. Sarfati, K. Jaton-Ogay, P. Ave, and J.-P. Latgé.** 1993. Virulence of alkaline protease-deficient mutants of *Aspergillus fumigatus*. FEMS Microbiol. Lett. **106:**39–46.
17. **Okada, N., C. Sasakawa, T. Tobe, M. Yamada, S. Nagai, K. A. Talukder, K. Komatsu, S. Kanegasaki, and M. Yoshikawa.** 1991. Virulence-associated chromosomal loci of *Shigella flexneri* identified by random Tn*5* insertion mutagenesis. Mol. Microbiol. **5:**187–195.
18. **Palmer, L. M., C. Scazzocchio, and D. J. Cove.** 1975. Pyrimidine biosynthesis in *Aspergillus nidulans*. Isolation and characterisation of mutants resistant to fluoropyrimidines. Mol. Gen. Genet. **140:**165–173.
19. **Sandhu, D. K., R. S. Sandhu, Z. U. Khan, and V. N. Damodaran.** 1976. Conditional virulence of a *p*-aminobenzoic acid-requiring mutant of *Aspergillus fumigatus*. Infect. Immun. **13:**527–532.
20. **Scazzocchio, C.** Personal communication.
21. **Schaffner, A.** 1985. Therapeutic concentrations of glucocorticoids suppress the antimicrobial activity of human macrophages without impairing their responsiveness to gamma interferon. J. Clin. Invest. **76:**1755–1764.
22. **Schaffner, A.** 1992. Host defense in aspergillosis, p. 98–112. *In* J. E. Bennett, R. J. Hay, and P. K. Peterson (ed.), New strategies in fungal disease. Churchill Livingstone, Edinburgh.
23. **Schaffner, A., H. Douglas, and A. Braude.** 1982. Selective protection against conidia by mononuclear and against mycelia by polymorphonuclear phagocytes in resistance to *Aspergillus*. Observations on these two lines of defense in vivo and ex vivo with human and mouse phagocytes. J. Clin. Invest. **69:**617–631.
24. **Sherman, F.** 1991. Getting started with yeast. Methods Enzymol. **194:**3–21.
25. **Tang, C. M., J. Cohen, and D. W. Holden.** 1992. An *Aspergillus fumigatus* alkaline protease mutant constructed by gene disruption is deficient in extracellular elastase activity. Mol. Microbiol. **6:**1663–1671.
26. **Tang, C. M., J. Cohen, T. Krausz, S. van Noorden, and D. W. Holden.** 1993. The alkaline protease of *Aspergillus fumigatus* is not a virulence determinant in two murine models of invasive pulmonary aspergillosis. Infect. Immun. **61:**1650–1656.
27. **Tang, C. M., J. M. Smith, H. N. Arst, and D. W. Holden.** 1994. Virulence studies of *Aspergillus nidulans* mutants requiring lysine or *p*-aminobenzoic acid in invasive pulmonary aspergillosis. Infect. Immun. **62:**5255–5260.

*Editor*: D. H. Howard